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MGMT function determines the differential response of ATR inhibitors with DNA-damaging agents in glioma stem cells for GBM therapy

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Abstract

Background. The most prevalent cancer treatments cause cell death through DNA damage. However, DNA damage response (DDR) repair pathways, initiated by tumor cells, can withstand the effects of anticancer drugs, providing justification for combining DDR inhibitors with DNA-damaging anticancer treatments.

Methods. Cell viability assays were performed with CellTiter-Glo assay. DNA damage was evaluated using Western blotting analysis. RNA-seq and single-cell level expression were used to identify the DDR signatures. In vivo, studies were conducted in mice to determine the effect of ATris on TMZ sensitization.

Results. We found a subpopulation of glioma sphere-forming cells (GSCs) with substantial synergism with temozolomide (TMZ) using a panel of 3 clinical-grade ataxia-telangiectasia- and Rad3-related kinase inhibitors (ATRis), (elimusertib, berzosertib, and ceralasertib). Interestingly, most synergistic cell lines had O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation, indicating that ATRi mainly benefits tumors with no MGMT repair. Further, TMZ activated the ATR-checkpoint kinase 1 (Chk1) axis in an MGMT-dependent way. TMZ caused ATR-dependent Chk1 phosphorylation and DNA double-strand breaks as shown by increased γH2AX. Increased DNA damage and decreased Chk1 phosphorylation were observed upon the addition of ATRis to TMZ in MGMT-methylated (MGMT-) GSCs. TMZ also improved sensitivity to ATRis in vivo, as shown by increased mouse survival with the TMZ and ATRi combination treatment.

Conclusions. This research provides a rationale for selectively targeting MGMT-methylated cells using ATRis and TMZ combination. Overall, we believe that MGMT methylation status in GBM could serve as a robust biomarker for patient selection for ATRi combined with TMZ.

Key Points

1. ATR inhibitors sensitize TMZ activity in MGMT-methylated tumors.
2. ATR inhibitors interfere with ATR/CHK1 signaling, thereby mediating increased DNA damage.
3. MGMT methylation status could serve as a biomarker for patient selection using ATR inhibitors with TMZ.

Importance of the Study

Resistance to standard treatment, particularly to DNA-damaging therapies, is frequently observed in GBM patients. Aberrant DNA damage repair pathways can limit the therapeutic response to the standard treatment. This study reports that 3 clinical-grade ATRis (elimusertib, berzosertib, and ceralasertib) showed synergistic activity with temozolomide (TMZ) in a subpopulation of GSCs. Interestingly, most synergistic cell lines had MGMT promoter methylation, indicating that ATRi mainly benefits tumors with a lack of MGMT repair. Further, TMZ activated the ATR/Chk1 axis in an MGMT-dependent way. A mechanistic investigation revealed that TMZ caused ATR-dependent Chk1 phosphorylation and double-strand DNA damage which was increased by ATRi treatment. TMZ and elimusertib combination treatment also improved mouse survival as compared to TMZ alone treatment. This investigation provides a foundation for selectively targeting MGMT-methylated cells/tumors using ATRis and TMZ combination treatment for GBM. In addition, MGMT methylation status could serve as a robust biomarker for patient selection for ATRi combined with TMZ.

Glioblastoma (GBM) is an incurable tumor, with a median overall survival duration of around 15 months with the best standard of care.[1] There is an unmet need to develop effective therapies for GBM. The short-term response rates to chemotherapy have greatly improved, and patients often experience relapse, with refractory, resistant, and incurable GBM and a poor survival expectancy.[2] Despite advances in cancer genome sequencing and the development of many targeted therapies, understanding the relationship between the tumor profile and therapeutic response remains a major obstacle to translating existing drugs into effective treatments because of GBM's heterogeneity. The development of clinical resistance to many therapies is a function of marked tumor heterogeneity and cellular adaptation to therapeutic pressure.

Temozolomide (TMZ) displays antitumor activity and limited toxicity; however, it results in a survival benefit of only 2.5 months because of the rapid occurrence of treatment resistance and tumor relapse.[3–7] Inhibition of TMZ-induced DNA damage repair response represents an attractive strategy for potentiating the cytotoxic effects of TMZ.[8] TMZ cytotoxicity is mediated by the addition of methyl groups at N7 and O6 sites on guanines and the O3 site on adenines in genomic DNA.[9] In approximately 60% of patients, O6-methylguanine (O6-MetG) is rapidly removed by the enzyme O6-MetG-DNA methyltransferase (MGMT), conferring resistance to chemotherapy (MGMT + unmethylated). MGMT plays a key role in repairing O6-site lesions induced by lomustine and carmustine, the other standard alkylating agents for GBM.[10] Understanding the underlying mechanism of MGMT-mediated repair and modulating MGMT activity in these patients is critical to enhance TMZ/lomustine/carmustine-mediated chemotherapy.[11] In the remaining 40% of patients, MGMT protein expression is absent because of methylation of MGMT promoter (MGMT–, methylated) and MGMT-mediated repair of O6-MetG is deficient, and cells use a detour pathway to maintain genomic stability. The unrepaired O6-MetG leads to stalled replication forks that result in DNA double-strand breaks, which are repaired by 2 major mechanisms: nonhomologous end-joining and homologous recombination. Ataxia-telangiectasia-mutated (ATM) serine/threonine protein kinase and Rad3-related (ATR) signaling is activated to repair single-ended and double-stranded breaks by homologous recombination. Several key molecules of DNA damage repair (ATM, ATR, RAD51, and DNA-PK) in homologous recombination and the nonhomologous end-joining-dependent DNA repair pathway have been reported to be involved in resistance to alkylating agents.[12–15]

The ATR-Chk1 pathway, the primary effector of replication checkpoints and DNA damage, prevents damaged cells from beginning mitosis.[16,17] ATR controls cell cycle checkpoints, replication fork stability and restart, as well as origin firing after activation. However, few studies have outlined the precise processes by which ATR triggers these reactions. The checkpoint reactions that are caused by ATR-activated Chk1 are those with the best-characterized mechanisms. One of these is the G2 checkpoint, which inhibits entry into mitosis when there is damaged DNA present via controlling the CDC25 phosphatase.[18] ATR is of special importance in GBM biology because it plays a key function in shielding GBM cells from TMZ.[19] As with other DNA damage response (DDR) inhibitors, there is significant concern about ATR inhibitors' (ATRis) toxicity in noncancerous cells, as it is required for the survival of many cell types.[20]

In this report, we provide evidence of MGMT-dependent synergistic interaction between ATRis and TMZ. We discovered that TMZ causes double-strand breaks and activates the Chk1 axis in tumor cells with methylated MGMT promoter (MGMT–). According to these findings, MGMT is a key molecular biomarker for the ATRi and TMZ combination. Notably, this study serves as the foundation for the development of a therapeutically effective DNA repair inhibitor and DNA-damaging agent combination treatment for GBM.

Materials and Methods

Cell Lines and Reagents

The GSC lines were established by isolating neurosphere-forming cells from fresh surgical specimens of human GBM tissue obtained from patients at The University of Texas MD Anderson Cancer Center from 2005 to 2008, as described previously.[21] Eight GSC lines [4 with MGMT-unmethylated/MGMT expression (MGMT+)] and [4 with MGMT-methylated/no MGMT expression (MGMT-)] were

cultured in DMEM/F12 medium containing B27 supplement (Invitrogen), basic fibroblast growth factor, and epidermal growth factor. Short tandem repeats using the Applied Biosystems AmpFISTR Identifier kit were used to authenticate cells. The last authentication test was performed in July 2017. All cell lines tested negative for mycoplasma contamination using the MycoAlert Detection Kit.

TMZ was from Sigma–Aldrich and lomeguatrib, elimusertib, ceralasertib, and berzosertib were from Selleckchem. For in vitro use, all inhibitors were dissolved in dimethyl sulfoxide. For irradiation experiments, we performed photon irradiation using an X-RAD 320-Precision X-Ray Biological Radiator to deliver a precise dosage of 2 Gy to 12 Gy to the cultured cells. After irradiation, cells were transferred to a cell culture–grade incubator for downstream applications. Sham-treated cultures were kept in close proximity to the X-RAD device for the same amount of time without exposure to X-rays.

Cell Viability Assay

For the cell viability assay, 5000 cells per well were plated in 96-well plates and treated in triplicate for 5 days with 10nM–2μM of elimusertib, berzosertib, and AZ6738. For TMZ synergy with elimusertib, different dosages of TMZ ranging from –50 μM to 300 μM were each combined with various dosages of elimusertib ranging from 0.25 μM to 2.25 μM for 72 h. For radiation synergy with elimusertib, different dosages of ionizing radiation from 2Gy to 8Gy were each exposed to various dosages of elimusertib from 50 nM to 200 nM for 24 h. In addition, 3—12 Gy of ionizing radiation was combined with 0.25 μM–2.25 μM dosages of elimusertib for 72 h. Cell proliferation was estimated using the CellTiter-Glo or CellTiter-blue viability assay kit (Promega). The IC_{50} values were calculated as the mean drug concentration required to inhibit cell proliferation by 50% compared with vehicle-treated controls. Drug synergy between TMZ or radiation therapy with elimusertib was measured using the software Combenefit, and the graphs were calculated using LOEWE Additivity.[22]

Colony Assay

Cells were pretreated with the 150 μM of TMZ in culture for 72 h in T25 flasks and then seeded in 6-well plates in triplicate wells, with a 3-fold dilution ranging from 9000 to 37 cells per well. Within 1 h after seeding, cells were treated with the 50 nM of elimusertib and placed in the incubator for 14 days. Fresh media was added when necessary during the 14 days to maintain the colonies. Colonies were counted using the (Cell3iMager Screen), and colonies above 150 μm were counted for analysis.

Cell Growth Curve

For the growth curve, 200 000 cells per well were plated in 12-well plates and treated in duplicate with 10 Gy of ionizing radiation using the X-RAD 320-Precision X-Ray Biological Radiator. One hour after radiation therapy, 50 nM of elimusertib was added under the indicated conditions and the same dosage of DMSO was added to the controls. Twenty-four and 72 h after radiation therapy, the drug media was removed and replaced with fresh media. The number of cells in each well was estimated using Vi-Cell counter (Beckman Coulter) and counted 0, 3, and 7 days after the drug media was removed.

Immunoblotting Analysis

Cells were harvested in lysis solution, and the extracted proteins were subjected to immunoblotting, as described previously,[23] using the following primary antibodies: antiphospho-ATR (phosphor-T1989), antiphosphoChk1(phosphor-S296), anti-Chk1, anti-cleaved PARP, and anti-γH2AX (phosphor-S139) (Cell Signaling); anti-ATR (Santa Cruz Biotechnology); and anti-MGMT (Sigma). GAPDH antibody was purchased from Sigma and used as a loading control.

RNA-Seq and Gene Set Enrichment Analysis Data Analysis

GSCs were subjected to total RNA isolation using the RNeasy kit (Qiagen) and quantified using NanoDrop. For the gene set enrichment analysis, the RNA-seq read counts for GSCs with synergistic ($n = 3$) and antagonistic ($n = 3$) phenotypes were normalized by EdgeR (Galaxy Version 3.24.1 + galaxy1). The normalized log2(CPM) values for all genes across 2 groups of GSCs were evaluated for enrichment of gene sets. Specifically, enrichment of 6 of 7 gene sets focused on DNA damage, repair, and response was observed in GSCs with an antagonistic phenotype, where 3 gene sets (DNA-damage response signal transduction, DNA integrity checkpoint, and DNA damage checkpoint) had a false discovery rate (FDR) < 25%.

For pathway analyses, DESeq2 (Galaxy Version 2.11.40.6 + galaxy1) identified the differentially expressed genes between synergistic (GSC:20, 262, 272, and 275), and antagonistic (GSC: 6-27, 811, 11, and 274) GSCs. Briefly, the RNA-seq read counts were preprocessed by RUVSeq (Galaxy version 1.16.0) to remove unwanted variation; the resulting batch effects were then factored to determine differentially expressed genes. The MA-plot shows the log2 fold changes that were attributable to a given variable over the mean of normalized counts across all samples. The points are highlighted (colored red) if the adjusted *P* value is < .1. Single-cell RNA-Seq (sc-RNA-seq) analysis for DDR genes was done using the Broad Institute single-cell portal (SCP; https://singlecell.broadinstitute.org/single_cell) web platform. The single-cell level expression of DDR signatures was analyzed in human glioma tumors from a public dataset from the Abdelfattah et al. study (GSE182109).[24]

In Vivo Experiments

To test the in vivo efficacy of ATRis, we studied the effect of TMZ and elimusertib in an intracranial mouse model. For that, we first created luciferase-expressing (MGMT-) GSC262 for in vivo imaging, cells were transfected with MSCV-Luciferase-EF1-copGFP-T2A-Puro BLIV

2.0 Lentivector (SBI System Biosciences). For intracranial models, (MGMT–) luciferase-expressing cells (0.5×10^6) were implanted intracranially into 4- to 6-week-old nu/nu mice using a previously described guide-screw system.[25] Animals were then randomly divided into 4 groups of 5 mice in each group. Two cycles of sequential treatment were administrated. For each cycle, TMZ (50 mg/kg/day for 5 days) was given by oral gavage; ATR inhibitor elimusertib at 50 mg/kg/day for 3 days/week was given the following week for 4 weeks by oral gavage. Tumor growth was visualized and quantified using the IVIS Spectrum in vivo imaging system. Mice were monitored daily and euthanized when they became moribund. Whole tumors were extracted, rapidly frozen in liquid nitrogen, and stored at –70°C.

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism software. Survival curves were plotted using the Kaplan–Meier method, and log-rank tests were used to compare survival curves between groups. Comparisons between 2 groups were performed using a Student's *t*-test; comparisons between more than 2 groups were analyzed by one-way analysis of variance (ANOVA) with corresponding Tukey's multiple comparison tests. If not indicated otherwise, analyses of significance were performed using 2-tailed tests, and $P < .05$ was considered statistically significant. Drug synergy was determined using Combenefit software.

Study Approval

The animal study was approved by the institutional review board and Institutional Animal Care and Use Committee of MD Anderson.

Results

ATRis Causes Sensitization of MGMT-Methylated (MGMT−) GSCs to TMZ

To test the ATRis capacity to sensitize TMZ in GSCs, we first investigated the effects of 3 ATRis- elimusertib, berzosertib, and ceralasertib on 4 (MGMT+) GSC lines and 4 (MGMT–) GSC lines; cell survival was assessed using the CellTiter-Glo assay. (MGMT–) cells were resistant to all 3 ATRis as monotherapy, (Figure 1A). Next, we tested 3 (MGMT+) cell lines and (MGMT–) cell lines with the combination of TMZ and the ATRi, elimusertib for combination effect. We observed TMZ and elimusertib synergy in (MGMT–) cells (Figure 1B). However, TMZ and elimusertib did not synergize in (MGMT+) cells, signifying that MGMT methylation status is important for synergistic activity between TMZ and ATRis.

To validate the results of the primary screening, we further tested GSCs with additional ATRi, berzosertib, and ceralasertib; all 3 methylated cell lines showed synergy with TMZ, as indicated by green, whereas all unmethylated cell lines showed no synergy with TMZ or ATRis, as shown by red or orange (Figure 1C). Taken together, these findings indicate that MGMT silencing is necessary for the synergistic activity between TMZ and ATRis.

MGMT Expression Is Associated with Synergistic Tumor-Cell Killing

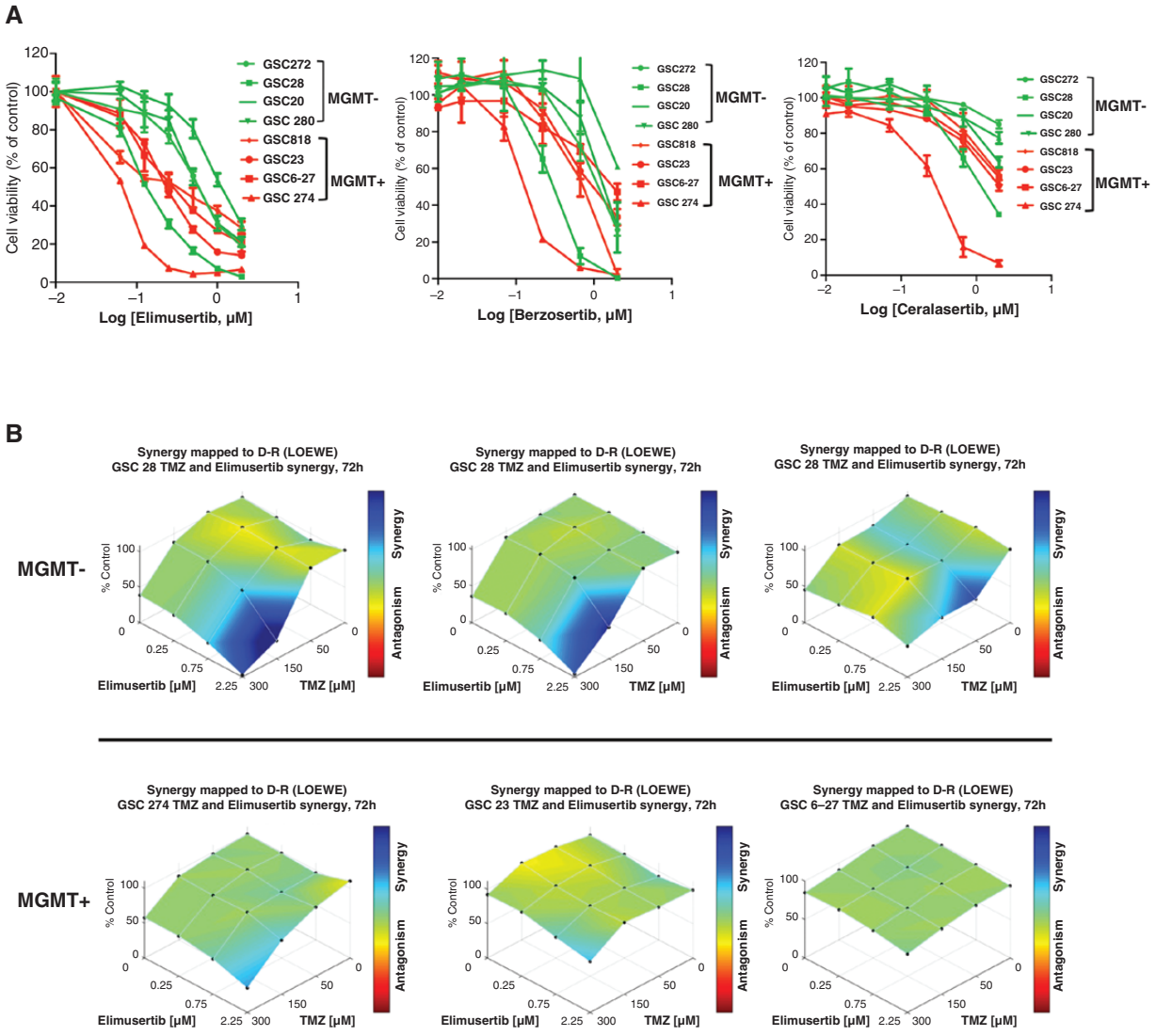
Using TMZ and elimusertib, we conducted a clonogenic long-term survival analysis. (MGMT–) GSCs demonstrated sensitivity to TMZ alone, and we observed a marginal improvement in the self-renewal capacity of the GSCs with the combination of TMZ and elimusertib (Supplementary Figure S1A and S1C). This sensitivity was observed in the size and number of the colonies (Supplementary Figure S1A and S1C). All of the (MGMT+) cells were resistant to TMZ therapy; they also did not respond to the combination of TMZ and elimusertib, as shown by micrographs and a bar graph (Supplementary Figure S1B). Thus, TMZ had a limited self-renewal effect with ATRis in (MGMT–) GSCs in clonogenic survival assays.

Because of the role of ATRis as radiosensitizing agents, we evaluated the effectiveness of ionizing radiation and ATRi elimusertib combined in both MGMT+ and MGMT– GSCs and unmethylated GSCs. The combination of radiation therapy and ATRis did not sensitize GSCs, as radiation alone was very effective at decreasing the proliferation of cells (Figure 2A, B). We also tested the ATR/Chk1 signaling pathway alteration after treating cells with radiation and ATRi. We did not observe any discrimantion between MGMT+ and MGMT– GSCs (Figure 2C). Moreover, no synergy was observed in any cell type, irrespective of MGMT status indicating that this combination is not effective in tumors/cells that respond well to ionizing radiation.

RNA-Seq Identified High Expression of DDR in GSCs

To understand the precise mechanism of ATRi activity, we selected synergistic (GSC:20, 262, 272, and 275) and antagonistic (GSC: 6-27, 811, 11, and 274) GSC lines and total mRNA was subjected to RNA-seq analysis. We conducted a differential expression analysis using DEseq2 and the read counts from RNA-seq. The analysis showed that the expression of hundreds of differentially altered genes that are elevated and downregulated in synergistic and antagonistic GSCs (Figure 3A and Supplementary Table). DESeq2 (Galaxy Version 2.11.40.6 + galaxy1) identified the differentially expressed genes between synergistic and antagonistic GSCs. The MA-plot shows the log2 fold changes that were attributable to a given variable over the mean of normalized counts across all samples. The points are highlighted (colored red) and the adjusted *P* value is < .1. Figure 3A depicts an MA-plot, showing the genes (as red dots) with significantly altered expression levels in a synergism versus antagonism groups (Figure 3A).

We ran a rank-based gene set enrichment analysis on differentially expressed genes from RNA-seq data in synergistic and antagonistic cell lines, which revealed enrichment of the DNA integrity checkpoint (NES = –1.46642,



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	Cell Lines	TMZ	Elimusertib+TMZ	Ceralasertib+TMZ	Berzosertib+TMZ
MGMT–	GSC272	146	19	84	83
	GSC28	210	1	3.6	33
	GSC280	214	7	55	27
MGMT+	GSC818	470	110	299	399
	GSC23	369	362	778	91
	GSC274	389	550	275	186

Figure 1. ATRis selectively potentiate anti-proliferative activity of TMZ in [MGMT–] methylated GSCs. (A) MGMT-methylated and MGMT + unmethylated GSCs were treated with 10 nM–2 μM ATRis elimusertib, berzosertib, and ceralasertib for 3 days; cell viability was assessed using a CellTiter-blue assay. Plots of dose–response curves were created in triplicate plates. Green indicates MGMT-methylated GSC lines and red indicates MGMT-unmethylated GSCs. (B) Synergy experiments were performed with TMZ and elimusertib, and synergy was assessed using Combenefit software in both (MGMT+)-methylated and [MGMT–]-unmethylated GSCs (n = 3). The color key: blue indicates synergy, whereas orange/red indicated anatagonism. (C) Screening of 3 ATRis shows synergistic action with TMZ, as shown by the IC_{50} in 6 GSCs treated with combination of TMZ and ATRis, elimusertib, berzosertib, and ceralasertib for 72 h. The color intensity (green to red) indicates the proporatinal IC_{50} values of various treatment conditions in GSCs. More green means lower IC_{50} and red means higher IC_{50}.

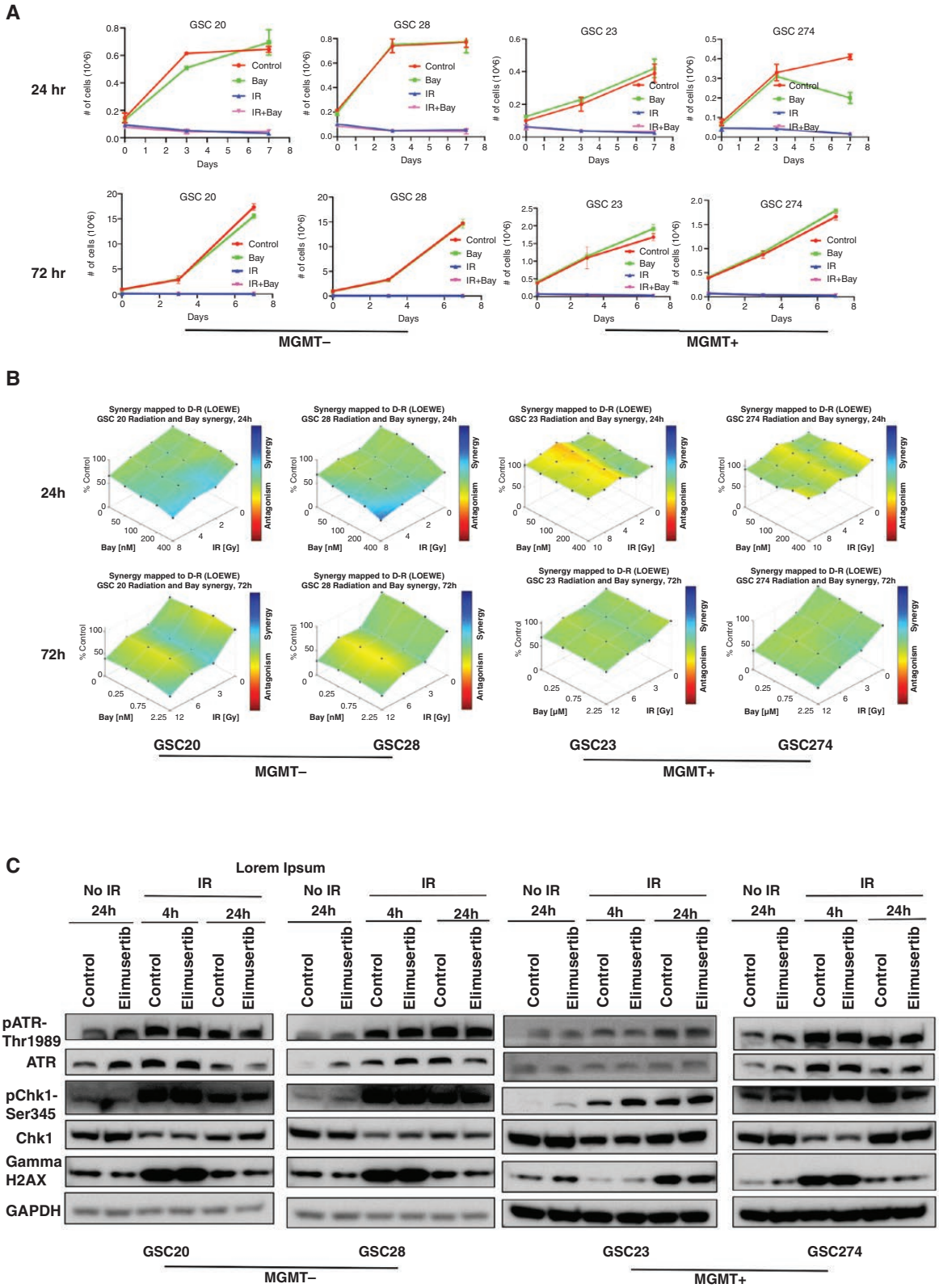

Figure 2. Radiation therapy did not sensitize GSCs to ATRis: (A) GSCs were treated with 10 Gy of ionizing radiation for 1 h and then 50 nM elimusertib. The numbers of cells in each well were counted 24 and 72 h after radiation therapy, and 0, 3, and 7 days after the drug media was removed. (B) For synergy studies, cells were irradiated with 0–12 Gy of ionizing radiation, followed by treatment with 50 nM to 2.25 μM ATRis elimusertib, berzosertib, and ceralasertib for 3 days; cell viability was assessed using a CellTiter-Glo assay. Drug synergy between radiation and elimusertib was measured using the software Combenefit, and the graphs were calculated using LOEWE Additivity. (C) Western blot of [MGMT+] and [MGMT– cells showing ATR activation through ATR/pChk1 signaling after 10 Gy of radiation treatment for the time course (4 h and 24 h after radiation treatment) indicated. Elimusertib (50 nM) was added 1 h after radiation, and the lysate was collected after the time course indicated.

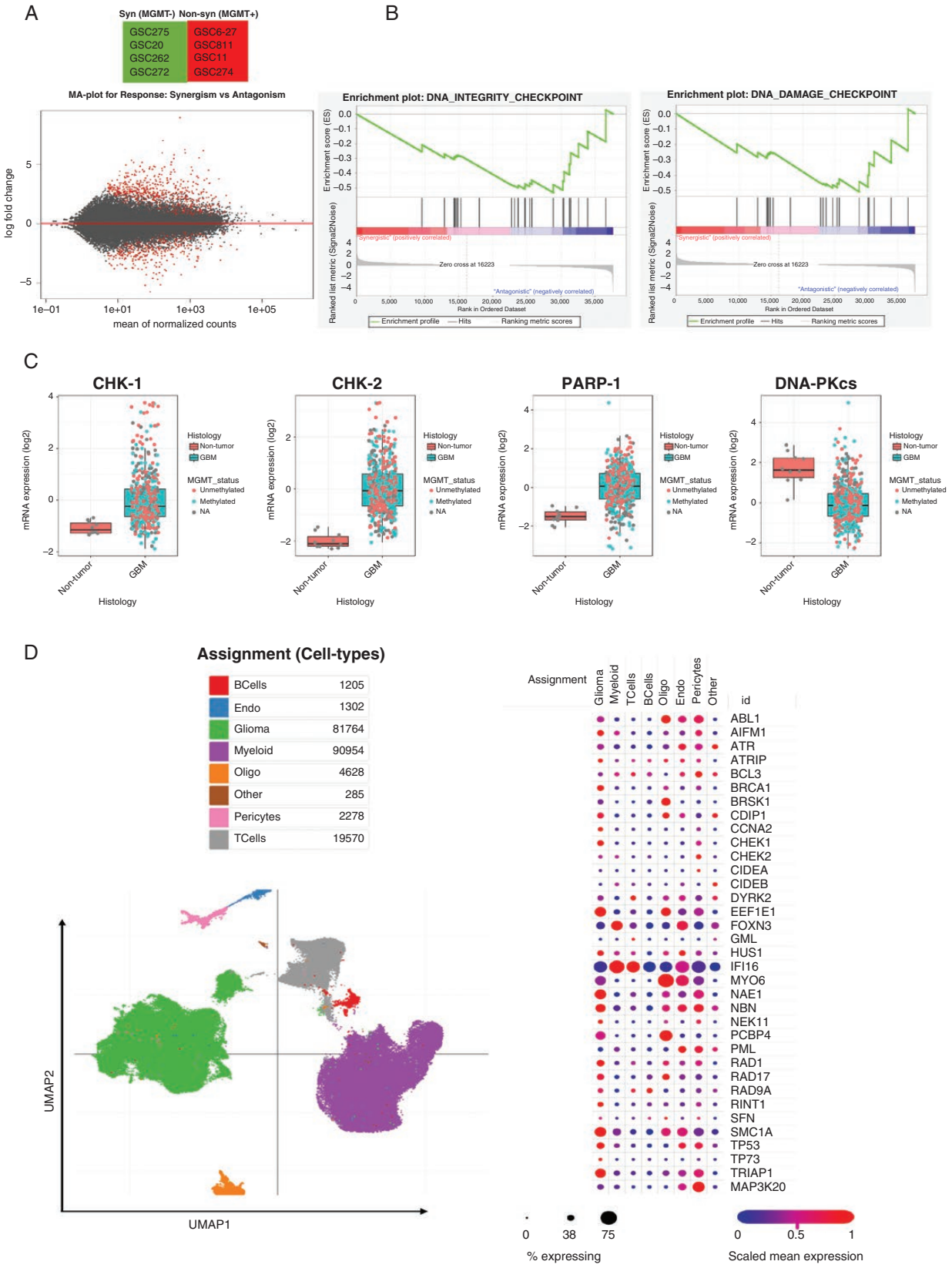

Figure 3. Increased DNA damage repair capacity in resistant cells. (A) An MA-plot shows the genes with significantly altered expression levels in a synergitic (GSC:20, 262, 272, and 275), vs. antagonistic (GSC: 6-27, 811, 11, and 274) groups. The gene expression data visualized as a two-dimensional scatter plot of the log2 ratio of expression values between the synergistic vs. antagonistic cell lines. MA plots show the log-fold

change (y-axis) (M-values, ie, the log of the ratio of level counts for each gene between two cell types against the log-average (x-axis), A-values, ie, the average level counts for each gene across the samples). Each dot represents one gene, and the red color indicates the genes identified to be differentially expressed between the synergistic vs. antagonistic cell lines using a false discovery rate (FDR) of <0.05. (B) GSEA of differentially expressed genes between synergistic and antagonistic cell lines showing enrichment of the DNA integrity checkpoint (NES = –1.46642, FDRq value of 0.138012) and DNA damage checkpoint (NES = –1.46267, FDRq value of 0.108772) in synergistic cell lines compared to in resistant cells with associated upregulated gene sets. The x-axis represents genes, ordered by expression changes between two cell lines. The cumulative enrichment score is represented on the y-axis. (C) Bar graphs show expression of CHK1, CHK2, PARP1, and DNA-PKcs in TCGA microarray (10 nontumors and 528 glioblastomas) datasets analyzed using the GlioVis platform. The expression of these genes was further classified into methylated and unmethylated tumors shown as red and green dots. (D) Showing the various cluster for different cell-types in human glioma and the scRNA-seq analysis of the DDR signatures in tumor and nontumor cells. The single-cell level expression of DDR signatures was analyzed in glioma tumors from public dataset from Abdelfattah et al. study (GSE182109).

FDRq value of 0.138012) and DNA damage checkpoint (NES = ––1.46267, FDRq value of 0.108772) in synergistic cell lines compared to antagonistic cells with associated upregulated gene sets (Figure 3B) and the list of some of the genes are listed in Supplementary Figure 2.

We further evaluated DDR gene expression in glioma patients in the TCGA datasets using the GlioVis platform. We found high expression of Chk1, Chk2, poly(ADP-ribose) polymerase-1 (PARP1), and DNA-dependent protein kinase (DNA-PKcs) in glioma patients (Figure 3C) providing an opportunity for combination treatment.

To further understand the DDR genes expression in a cell-type-specific manner in human glioma tumors, we analyzed 201 986 cells (malignant, immune, and other stromal cells) from 18 glioma patient tumor samples at the single cell using the public dataset of sc-RNA-seq. Our sc-RNA-seq analysis showed that several DDR genes, particularly CHEK1, BRCA1, NAE1, and TRIAP1, are upregulated in only malignant/tumor (glioma) cells (Figure 3D). However, many genes are also expressed in nonmalignant cell types as well (Figure 3D).

TMZ Exclusively Stimulates the ATR-Chk1 Axis in (MGMT-)-Methylated GSCs

ATR controls the interaction between DNA damage, cell-cycle arrest, and cell death. We postulated that TMZ only activates the ATR axis in (MGMT–) cells in light of MGMT-dependent DNA damage and cell death after TMZ treatment. To measure ATR activity, we evaluated the levels of phosphorylated Chk1. The ATR-Chk1 axis was found to be activated in (MGMT-) GSC 20 and GSC 28 cells, with greater levels of ATR (Thr1989) and Chk1 (Ser345) phosphorylation after TMZ treatment (Figure 4A). ATRi treatment decreased this activation. No MGMT-dependent difference in pChk1 levels resulted from treatment with TMZ in (MGMT+) unmethylated cells (GSC 23 and GSC 274).

We further determined whether sequential treatment with TMZ and ATRi had an increased effect on the ATR/Chk1 signaling axis by pretreating cells with elimusertib for 24 h, followed by TMZ treatment (Figure 4B). We demonstrated that TMZ and ATRis, whether given as cotreatment or in succession, stimulate the ATR/Chk1 signaling axis in the same way, showing that similar activation of the ATR/Chk1 axis occurred with both concurrent and sequential treatment.

ATRi Enhanced DNA Damage and Apoptosis in (MGMT−) Methylated GSCs

Further, to look for increased DNA damage in (MGMT–) cells after TMZ treatment, we used γH2AX protein as a marker for double-strand breaks of DNA damage. We observed low and consistent amounts of γH2AX after TMZ treatment in (MGMT–) cells. However, we observed an increase in γH2AX expression after ATRi and TMZ combination treatment, indicating increased DNA damage and more double-strand breaks after combination treatment (Figure 4C). We further showed increased cleaved PARP after ATRi and TMZ combination treatment, indicating increased cell death. Together, these data suggest that MGMT plays a role in ATR signaling in TMZ-induced lesions, leading to increased DNA damage and cell death.

Brain-Penetrant ATRi elimusertib Increased TMZ Sensitivity in (MGMT-) Methylated Tumors in Vivo

To test the in vivo efficacy of ATRis, we studied the effect of TMZ and elimusertib in an intracranial mouse model. (MGMT–) methylated GSCs were implanted intracranially into nude mice. Mice were treated sequentially with TMZ and elimusertib (Figure 5A). Elimusertib treatment alone resulted in no significant survival benefit (Figure 5A); however, we observed enhanced survival in animals treated with the combination of TMZ and elimusertib (Figure 5B and 5C). The median survival duration was 62 days for elimusertib-treated mice, 73 days for TMZ-treated mice, and 59 days for vehicle-treated mice ($P = .0003$ for combination versus TMZ alone, $P = .0001$ for combination versus Elimusertib alone, and $P = .0019$ for combination versus control, log-rank). In contrast, the median survival duration of animals treated with the combination of TMZ and elimusertib was 87 days, indicating a 47% increase in survival duration compared to controls. The combination treatment also induced a significant tumor growth relative to TMZ alone or elimusertib alone in (MGMT–) methylated intracranial tumors using bioluminescence imaging, shown in the figure are images from days 24, 39, and 52. A tumor tissue analysis revealed that elimusertib treatment resulted in a decrease in pChk1, which was further decreased after combination treatment with TMZ (Figure 5D).

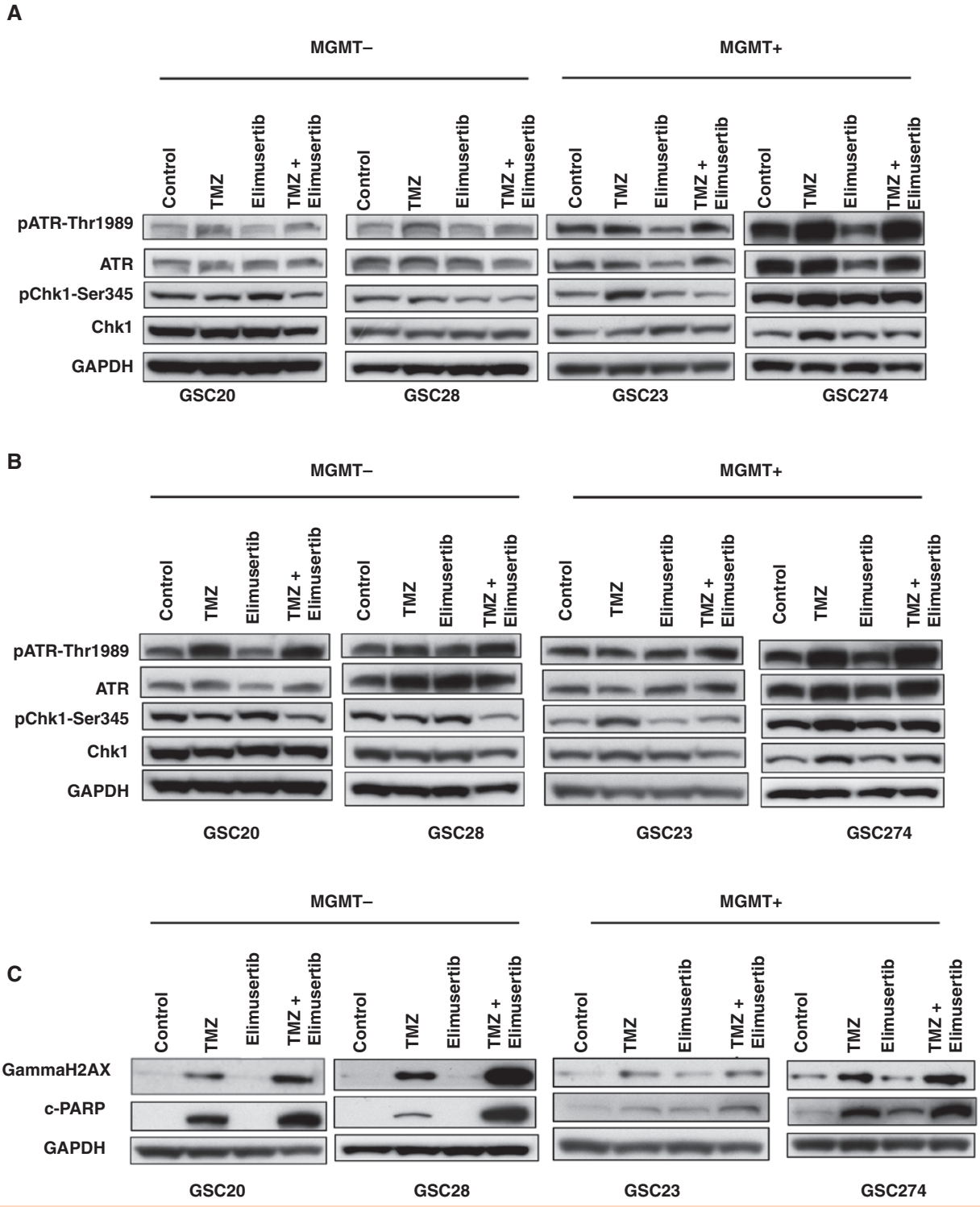

Figure 4. TMZ-induced DNA damage and apoptosis in MGMT-deficient cells via activating the ATR-Chk1 axis. (A) Effect of 50 nM elimusertib, 150 μM TMZ, and combination treatment for 48 h on levels of ATR, Chk1, and γ-H2AX in GSC20, GSC28, GSC23, and GSC274 cells by immunoblotting. GAPDH and total Chk1 were used as loading controls. Figure 4 is representative of four independent experiments. (B) Effect of sequential treatment with TMZ and elimusertib by pretreating cells with elimusertib for 24 h, followed by TMZ treatment for an additional 24 h on levels of ATR, CHK1, and γ-H2AX in GSC20, GSC28, GSC23, and GSC274 cells over time by immunoblotting. GAPDH and total Chk1 were used as loading controls. Figure is representative of four independent experiments. (C) Immunoblot analysis of the effect on levels of γ-H2AX (as measures of DNA damage) and cleaved-PARP (c-PARP, reflecting induction of apoptosis) in GSC20, GSC28, GSC23, and GSC274 cells after elimusertib, TMZ, and combination treatment. GAPDH was loaded as a control and figures are representative of four independent experiments.

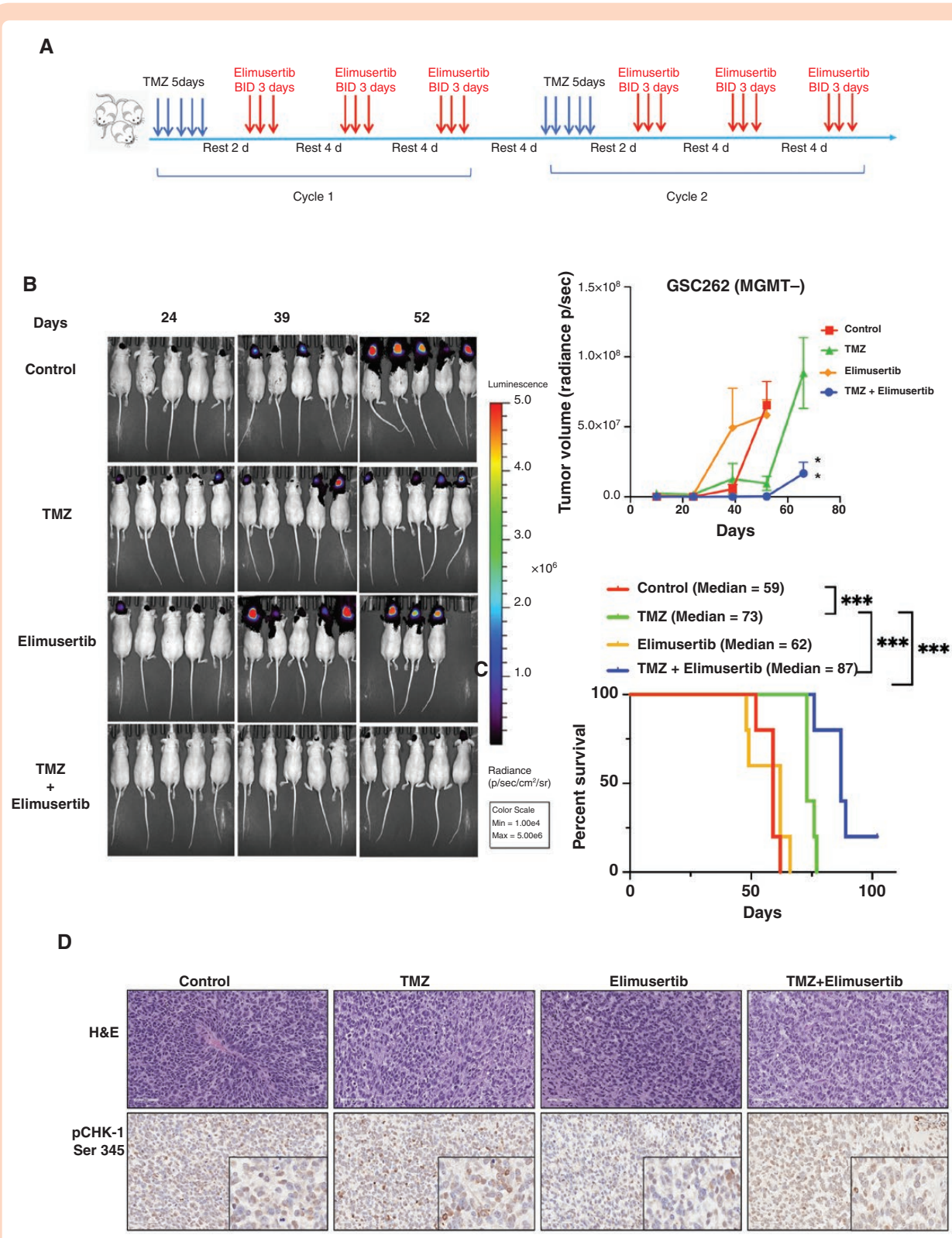

Figure 5. ATRi inhibiton by elimusertib restored TMZ sensitivity in (MGMT+) tumor in vivo. (A) graphic scheme for sequential combination of TMZ with elimusertib, TMZ 50 mg/kg/day; elimusertib 50 mg/kg/day. (B and C). Mice bearing (GSC 262, [MGMT–]) intracranial tumors were administrated with TMZ, elimusertib, or sequential combination. Tumor growth was evaluated by bioluminescence imaging (C), survival curves were compared using Kaplan–Meier survival plots ($P = .0003$ for combination vs. TMZ alone, $P = .0001$ for combination vs. Elimusertib alone, and $P = .0019$ for combination vs. control, log-rank). (D). The representative images (H&E) of mouse tumors and immunohistochemistry (IHC). For IHC, the tissue sections were incubated with antibody against pChk1 and developed using HRP-conjugated secondary antobody and DAB chromogen. Scale bars: 25 μm.

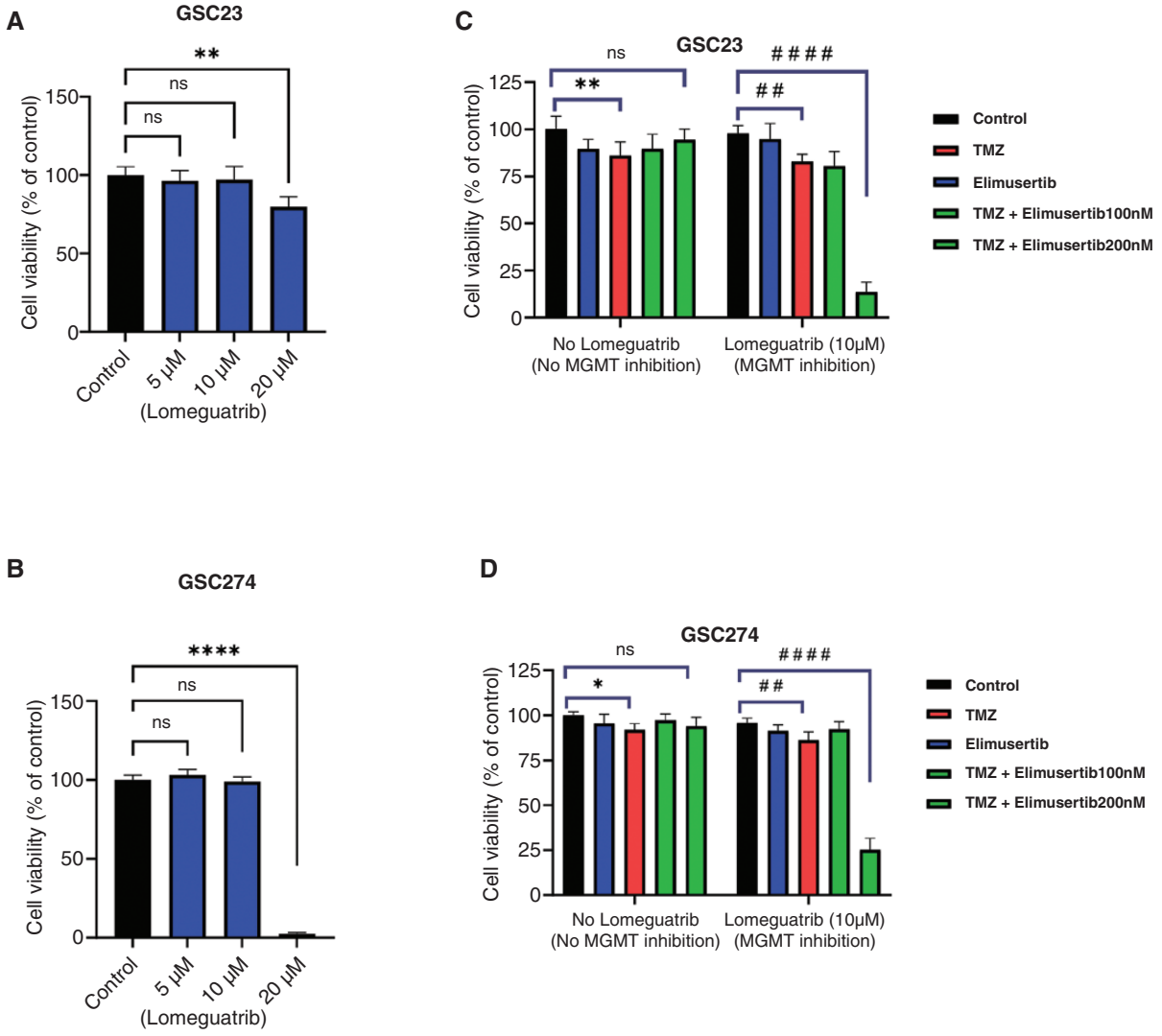

Figure 6. Lomeguatrib, a potent inhibitor of MGMT, sensitize the TMZ and ATRi combination treatment in unmethylated GSCs. (A and B) Bar graphs showed the cell viability of lomeguatrib (MGMT inhibitor) in GSC23 and GSC274. Cells treated with 5, 10, and 20 μM lomeguatrib for 3 days; cell viability was assessed using a CellTiter-glo assay. $^{**}P < .01$, $^{****}P < .0001$, and ns = not significant as compared to control. (C and D) The influence of pharmacological inhibition of MGMT on the sensitization of TMZ and ATRi (elimusertib) combination treatment in unmethylated GSCs. GSCs were pretreated with lomeguatrib (10 μM) for 24 h, cells were treated with TMZ (150 μM) alone and elimusertib (200 nM) alone as well as combination of TMZ and elimusertib (100 and 200 nM) for 48 h with and without lomeguatrib (MGMT inhibition). Data were normalized with untreated control and analyzed by 2-way ANOVA.$^{*}P < .05$, $^{**}P < .01$, and ns = not significant as compared to control (untreated), whereas $^{\#\#}P < .01$ and $^{\#\#\#\#}P < .0001$ as compared to control (lomeguatrib treated).

MGMT Inhibition Sensitized the TMZ and ATRi Combination in (MGMT+)-Unmethylated GSCs

To evaluate the MGMT-dependent specificity for the sensitization of TMZ activity to ATRis, we used lomeguatrib, a clinically tested, nontoxic, and potent pharmacological inhibitor of MGMT[26,27] to test potential increase in the TMZ + ATRi activity in (MGMT+)-unmethylated GSC lines. First, we treated the (MGMT+)-unmethylated GSCs (GSC 23 and GSC274) with 3 concentration (5, 10, and 20 μM) of lomeguatrib for 72 h to determine the nontoxic dose of this MGMT inhibitor in GSCs, and results showed that 5 and 10 μM of lomeguatrib did not induce any cytotocity in either of cell line (Figure 6A and 6B) However, high dose (20 μM) of lomeguatrib significantly decrease cell viability in both tested GSCs (Figure 6A and 6B). Finally, we used 10μM of lomeguatrib to test the effect of MGMT inhibition of TMZ + ATRi sensitization in (MGMT+) GSCs. GSC23 and GSC274 were pretreated with lomeguatrib for 24 h, then cells were cotreated with TMZ (150 μM) alone and elimusertib (200 nM) alone as well as a combination of TMZ and elimusertib (100 and 200 nM) for 48 h with and without lomeguatrib. The treatment of lomeguatrib (MGMT inhibition) significantly sensitized (increased cytotoxicity) of TMZ + elimusertib (200 nM) combination treatment in both the (MGMT+)-unmethylated GSCs (GSC 23 and GSC274)

as compared to control (lomeguatrib alone treated cells) (Figure 6C and 6D). However, cell exposed to similar treatment without lomeguatrib treatment (no MGMT inhibition) failed to sensitize the TMZ + elimusertib combination treatment in both cell lines (Figure 6C and D).

Discussion

Increasing evidence supports the notion that DDR signaling plays an important role in repairing DNA damage generated by conventional GBM therapy; hence, it has emerged as a molecular target for tumor-guided therapies. The use of DDR inhibitors to render GBM cells more vulnerable to conventional treatments is an urgent medical need for GBM patients.[13,14,28,29] One such DDR pathway, ATR, has been shown to enhance DNA repair and restore the integrity of the genome after TMZ treatment.[30–33] In this report, we show MGMT-dependent sensitivity to ATRis in a large set of clinically relevant GSC lines and ATRis' potential chemosensitization with TMZ in the treatment of MGMT– (methylated) GBM.

Screening of GSCs with a panel of 3 clinical-grade ATRis (elimusertib, berzosertib, and ceralasertib) showed that a subgroup of GSCs had significant synergism with TMZ but resistance to ATRi single-agent monotherapy. A further investigation showed that most of the synergistic cell lines were associated with MGMT promoter methylation, suggesting that only tumor cells with no MGMT repair will benefit from ATRi. We showed this synergy with numerous structurally distinct ATRis, and it was replicated in multiple cell lines. Therefore, 45–55% of GBM patients who have at least partial methylation of the MGMT promoter may benefit from the inclusion of TMZ and ATRis in their treatment regimen.

Consistent with our data, Jackson et al. demonstrated that treatment of (MGMT–) cells with TMZ increased their sensitivity to ATRis, both in vitro and in vivo, across numerous tumor cell types.[34] Radiation sensitization was insufficient to synergize GSCs with ATRis, indicating that TMZ activated the ATR axis in an MGMT-dependent manner.

Inhibiting ATR enhanced TMZ-mediated DNA damage, followed by decreased Chk1 phosphorylation. Treatment with ATRis and TMZ greatly elevated γH2AX, a marker of DNA double-strand breaks, in GSCs. This is because DNA replication in the S phase must proceed normally for the ATR-Chk1 pathway to function; when the ATR/Chk1 pathway is inhibited, DNA replication forks collapse and DNA double-strand breaks are produced.[35] Further, to avoid systemic toxicity, we showed that a sequential treatment strategy for combining DDRi with TMZ should be employed in in vivo models. We found that treating MGMT-deficient tumor cells with both TMZ and ATRis at the same time or sequentially led to increased DNA damage and apoptosis compared to either treatment alone. Taken together, our findings and those of a recent study indicate that inhibiting the ATR-mediated checkpoint permits TMZ-treated cells to continue DNA replication, increasing DNA damage and thereby leading to apoptosis.[36,37]

Given the high frequency of DNA damage pathway alterations in GBM, there have been focused efforts to pharmacologically target key enzymes, including PARP, DNA-PKcs, ATM, and ATR. The standard-of-care GBM treatment, ionizing radiation and alkylating chemotherapy, generates DNA damage that is repaired through the upregulation and activation of DDR enzymes.[38–41] Many DDR genes, such as ATM, ATR, alphathalassemia/mental retardation, X-linked, and DNA-PKcs, have been shown to enhance DNA repair and restore the integrity of the genome after TMZ treatment.[13,28,29]

It is generally acknowledged that the methylation state of the MGMT gene promoter serves as a predictive biomarker for treatment with the alkylator TMZ. The MGMT enzyme eliminates O6-metG lesions in the absence of promoter methylation. The O6-metG lesion activates the mismatch repair pathway in the presence of MGMT-promoter methylation (MGMT-), which works to heal the damage. ATR activation in response to TMZ therapy is modulated by the loss of MGMT expression caused by MGMT promoter silencing, which is linked to synergistic tumor cell death.[42–47] Our findings also confirm that the treatment of lomeguatrib (MGMT inhibition) significantly sensitized (increased cytotoxicity) of TMZ + elimusertib (ATRi) combination treatment in the (MGMT+)-unmethylated GSCs. However, cells exposed to similar treatment without lomeguatrib treatment (no MGMT inhibition) failed to sensitize the TMZ + elimusertib combination treatment in both cell lines. These findings indicated that MGMT-dependent specificity of the sensitization of TMZ activity to ATRis.

Earlier studies showed that TMZ induces senescence in glioma cells by arresting them in the G_2-M phase of the cell cycle that is initiated via ATR/Chk1-mediated degradation of CDC25c, leading to abrogated CDK1/cyclin B1 activity.[36] A study by Bindra et al. also showed the mechanistic understanding of how the mismatch repair proteins are involved in ATR activation by TMZ in (MGMT–) GBM cells.[48] Further studies have shown improved sensitivity to ATRis in vitro and in vivo in a variety of tumor cell types.[44,46] MGMT-promoter methylation status has been demonstrated in clinical studies to be able to predict the prognosis of glioma patients. Therefore, TMZ, not radiation therapy, is expected to produce a good therapeutic response in patients with MGMT-promoter methylation.

MGMT-promoter methylation of anaplastic oligodendroglioma tumors was found to be associated with improved overall survival and progression-free survival after radiation therapy or alkylating agents.[49] A pivotal 2005 trial in GBM patients with methylated MGMT promoter tumors responded better to therapy with TMZ than did other patients. The results of our previous studies have supported the combination of TMZ and PARP inhibitors in patients with MGMT-deficient tumors.[50,51] Thus, understanding the methylation landscape of the MGMT promoter in tumors can be essential for developing an effective treatment plan because only around 50% of malignancies express MGMT at low levels. Our findings revealed an accurate and efficient way to target MGMT-methylated tumors with ATRis and TMZ and form the basis of clinical trials to test their combination with TMZ using MGMT as a key molecular biomarker for patient selection.

Supplementary material

Supplementary material is available online at *Neuro-Oncology* (https://academic.oup.com/neuro-oncology).

Keywords

ATR inhibitors | glioblastoma | MGMT status | temozolomide

Conflict of interest statement

A.W.K.Y. serves as a consultant with DNATrix. The rest of the authors have no conflicts of interest to disclose.

Authorship statement

Designed the study: D.K., S.K., V.W.S.L.; S.W., R.R.T., and X.L.I.; conducted the experiment and analyzed the data: V.W.S.L; S.K., S.W., P.S., and X.L.; bioinformatic analysis: S.S.; wrote the main draft: D.K.; and review and editing: A.W.K.Y., S.K., F.F.L., and V.W.S.L. All authors read and approved the final manuscript.

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